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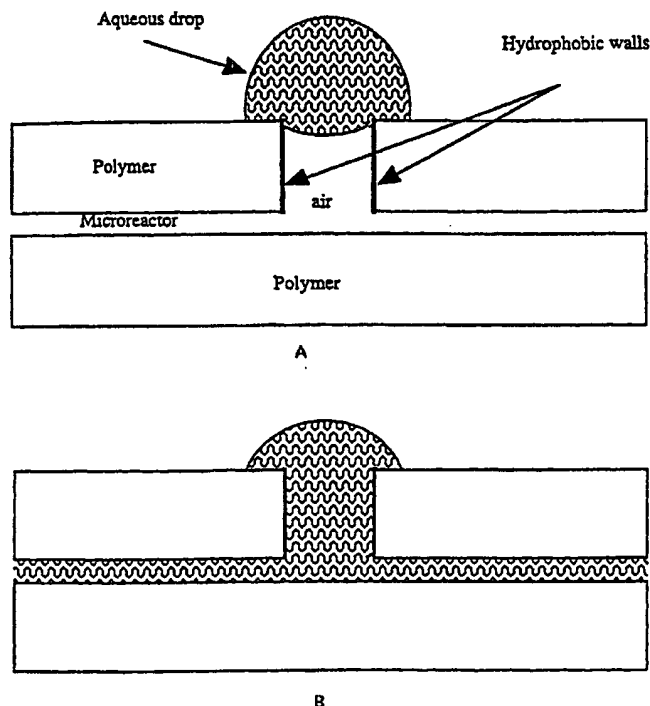
INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 7 : G01N 33/00	**A1**	(11) International Publication Number: **WO 00/58724**
		(43) International Publication Date: 5 October 2000 (05.10.00)

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(54) Title: MICROSCALE TOTAL ANALYSIS SYSTEM

(57) Abstract

The invention relates to an apparatus for performing chemical assays in an aqueous medium. The apparatus contains a reaction chamber(s) and a liquid in–flow channel connected to each chamber. The flow of liquid through the fluid in–flow channel to the reaction chamber is controlled by the presence of a hydrophobic inner surface on the walls of the in–flow channel. Under normal conditions fluid will not flow through the channel. However, application of an external force pushes the liquid through said channel into the reaction chamber. The invention is applicable to the monitoring of many different molecular interactions, in particular molecular recognition between an immobilised affinity partner and a species in solution, such as immunoglobulin/antigen interaction, DNA hybridisation, haptamer–protein interaction, drug and virus detection, high throughput screening of synthetic molecules and for determining the concentration and reaction kinetics of target species.

A

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MICROSCALE TOTAL ANALYSIS SYSTEM

This invention relates to apparatus for detecting the presence of a target species in an aqueous sample, and also to apparatus for determining the concentration and reaction
5 kinetics of target species. The invention is applicable to the monitoring of many different molecular interactions, in particular molecular recognition between an immobilised affinity partner and a species in solution, such as immunoglobulin/antigen interaction, DNA hybridisation,
10 haptamer-protein interaction, drug and virus detection and high throughput screening of synthetic molecules.

As many affinity complexations between two reaction partners are diffusion controlled, the time needed to reach reaction equilibrium is directly dependent on the mass
15 transport of the molecule. The diffusion time of a molecule in a solution is proportional to the square of the path length; typically a small molecule needs less than one second to diffuse through 10 μm while it needs two hours to traverse one millimetre. In order to decrease the
20 equilibrium time of the reaction, the chemical partners must therefore be placed as close as possible to each other; by reducing the reactor size to microdimensions, immobilising one partner on the surface of the reactor and filling the reactor with the second partner, the equilibrium time can be
25 dramatically decreased.

The use of microreactors not only enhances the speed of affinity assays, but also facilitates the obtaining of information concerning reaction kinetics, which is important in the understanding of the thermodynamic stability of
30 complexes. The affinity constant K_d is the ratio between the forward and reverse reaction rate constants k_+ and k_-, which represent the association and the dissociation constants respectively. A strong complexation is characterised by a very fast association and a very slow
35 dissociation, which in the particular case of sorbent affinity assays are adsorption and desorption from the

surface of the microreactor. The understanding of these thermodynamic properties can be used for the study of cross-reactivity between several antigens or of non-specific adsorption of a matrix element during an affinity assay. By modulating the incubation time of the solution, the complexation of higher affinity partners is favoured and the non-specific adsorption is then reduced to a minimum. This fact may be an important factor in the decrease of the detection limit in immunosorbent assays due to the minimisation of the background signal. This method can also be applied to monitor the adsorption of antigens of different molecular weight.

As the diffusion coefficient of a molecule is proportional to its mass, the diffusion time of the molecule through the reaction chamber is different for small and large molecules. In the case of molecules of different molecular weight with the same epitope, (e.g. fibrine degradation products), the K_d may be the same for all molecules, whereas the diffusion coefficient is different for each of them. When a kinetics experiment is carried out, the smaller molecules will quantitatively reach the antibodies before the larger ones. The monitoring of the signal resulting from the affinity reaction as a function of time can thus deliver useful kinetics information, which can help in understanding the degradation process. These kinetics events can be followed by modulating the residence time of molecules in contact with their reaction partners, which can most readily be achieved by immobilising an antibody on the walls of a series of microreactors and by incubating different solutions of the analyte of interest for different periods of time.

In the past, analytical procedures of the type described above (such as enzyme-linked immunosorbent assays - ELISAs) have been performed using microtiter plates and have been relatively slow. In recent years, great efforts have been made to reduce the size of analytical devices to micrometer scale with the effect of reducing reaction times.

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These miniaturised systems have been termed "microscale total analysis systems" (μ-TAS)[1], and they have already been recognised as convenient means of manipulating and analysing small sample quantities [2-8]. Most μ-TAS devices to date
5 have been produced by photolithography, wet chemical etching or thin film deposition on substrates such as glass, quartz and silicon [9,10]. In order to decrease production costs, plastic substrates have also been micromachined using either silicone rubber casting [11-14] injection moulding [15] embossing
10 [16,17] or laser photoablation [18]. These structures are planar devices with channels of micrometre size that are often sealed by thermal or anodic bonding to a glass cover. Interconnected channels may be fabricated easily, which makes possible the rapid separation and reactions in volumes
15 of few a picoliters. Other advantages of μ-TAS are the reduction of sample and reagent consumption and the increase of precision and reproducibility relative to bench scale apparatus [21,22].

Competitive immunoassays have also been performed on
20 micro-chips [23-25], but the micro-channels have only been used to electrophoretically separate free and bound forms of antigen or antibody. In these assays, antibody and labelled antigen are added in specific quantities to the sample to be analysed. The sample is then incubated with a
25 mixture of the labelled and native antigens that compete for a limited number of antibody binding sites. The micro-channel is then used to separate the free labelled antigen from the complex by capillary electrophoresis, and quantitation is performed by luminescence (fluorescence or
30 chemiluminescence) at the end of the separation channel. The amount of free labelled antigen measured is then related to the analyte concentration in the sample using a previously determined calibration curve. In this type of assay it is essential to avoid adsorption of a reaction partner on the
35 micro-channel walls.

Another type of immunoassay device has been developed for simultaneous analysis of multiple samples [26]. In this

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case, biotin-labelled antibodies are patterned onto an avidin-coated waveguide so as to form an array of six vertically oriented stripes of captured antibodies immobilised on the waveguide surface by avidin-biotin
5 bridges. Samples are then analysed using a sandwich immunoassay format by patterning another array of six horizontally oriented lines containing the corresponding fluorescent-labelled antigen at various concentrations. Fluorescent complexes on the surface of the waveguide are
10 then excited by a diode laser, and the fluorescence intensities of the 36 square dots is collected by a CCD camera. This immunosensor allows the analysis of multiple samples in parallel and simultaneous detection of more than one analyte per sample.

15   Numerous analytical methods utilise luminescence to detect an analyte of interest. Luminescence is the generic term referring to the emission of an electromagnetic radiation (UV, visible or IR) by an excited molecule that relaxes to its ground state, can be induced by
20 photoexcitation (photoluminescence) or by a chemical reaction (chemiluminescence and electrochemiluminescence). Chemifluorescence (CF) is another class of luminescent reactions which combines the reaction mechanisms of both PL and CL. In this case, a fluorogenic substrate A is converted
25 to a fluorescent product C by chemical reaction, and luminescence is generated by excitation of this product:

$$A + B \rightarrow C + D, \quad h\nu_1 + C \rightarrow C^* \quad \text{and} \quad C^* \rightarrow C + h\nu_2$$
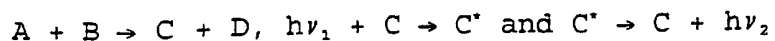
     For analytical purposes, one of the reactants of the assay system that is capable of generating luminescence can
30 be attached to a molecule in order to "label" it specifically. The presence or absence of an observable label attached to one or more of the binding materials is then used as an indicator of the existence of an analyte of interest. A large body of experiments has been developed to
35 detect and quantitate trace amounts of pharmaceuticals, microorganisms, hormones, viruses, antibodies, nucleic acids

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and other proteins by such methods. In clinical diagnostics for instance, competitive and sandwich immunoassays using luminescence detection are now used on a routine basis [27, 28].

In enzyme-mediated immunoassays, a molecule is labelled
5 with an enzyme that catalyzes the luminescence reaction. Typical examples are the detection of immunoreagents labelled with Horse Radish Peroxidase (HRP) or Alkaline Phosphatase (ALP) which, in the presence of hydrogen peroxide and hydroxide ions, respectively facilitate the
10 oxidation of luminol and dioxetanes and the hydrolysis of phosphate-containing reagents. Similarly, ALP has been used in CF assays to cleave a phosphate group from a fluorogenic substrate to yield a highly fluorescent product [29].

Luminescence assay methods are widely used in the
15 analysis of peptides, proteins, and nucleic acids. CL has been shown to be a highly sensitive detection method in both flow injection analysis (FIA) and high-performance liquid chromatography [30-32], and it has also been employed in capillary electrophoresis (CE) [33, 34] for the detection of
20 amino acids neurotransmitters [35], rare-earth metal ions [36] or labelled proteins [37]. However, it is in immunoassays that luminescence is the most commonly used detection method [27, 34, 38-43].

Among prior art methods for the measurement of
25 enzymatic reaction rate, US 4,621,059 discloses a method in which the light emitted by a luminescent substance flowing through a capillary column and reacting with an immobilised enzyme is collected through a plurality of optical fibers that are arranged along the longitudinal direction of the
30 column in order to determine the enzyme activity or the quantity of analyte of interest from the distribution of the luminescence intensity.

US 5,624,850 describes a method for performing immunoassays in capillaries in which fluorescence is used to
35 detect an analyte of interest in translucent capillaries

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having an inner diameter from ~0.1 μm to 1.0mm. Similarly,
homogeneous chemiluminescence immunoassays can be carried
out, for example as described in US 5,017,473, in which a
light absorbing material and a luminescent labelled tracer
5   are incubated with the analyte/anti-analyte complex, so that
all the emitted light is absorbed by the light-absorbing
material except that associated to the bound tracer.

A method is disclosed in US 5,585,069 in which two or
more samples are processed in parallel in a system
10 comprising a plurality of wells that are connected by one or
more channels to move a sample from one well to the other
using mechanical or electrokinetic pumping.   In this
apparatus, the channels are simply used as connections
between two wells, and are not used as reaction or detection
15 chambers.

One of the main difficulties associated with the use of
μ-TASs is the proper mixing of reagents, due to the low
Reynolds numbers of the flows. Another difficulty resides
in the accurate timing of fluid entry, which is essential
20 for kinetics studies.   In addressing these problems, the
applicants have found that fluid entry into a reaction
chamber (for example a microchannel) can be accurately be
controlled by means of a hydrophobic gate.

The present application therefore provides, in one
25 aspect, apparatus comprising: at least one reaction chamber;
at least one fluid inflow channel communicating with the or
each reaction chamber; and gate means adapted to prevent
passage of aqueous fluid through the fluid inflow channel(s)
into the reaction chamber(s), until such fluid is acted upon
30 by a fluid entry force; wherein the gate means comprises at
least a portion of the or each fluid inflow channel having
a hydrophobic inner surface.

Preferably, the apparatus has a plurality of reaction
chambers, which take the form of microchannels, each having
35 an associated fluid inflow channel.   Alternatively, a

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plurality of microchannels may be served by a single inflow channel, feeding into a common conduit communicating with the microchannels. In some preferred embodiments, the fluid entry force is provided by aspiration means connected to a
5 common conduit communicating with each microchannel at its end distal the inflow channel. In other embodiments, the apparatus comprises a rotatable support member and the fluid entry force is provided by centrifugal force upon rotation of the substrate. Conveniently, the support member may form
10 the substrate of the microchannel apparatus, with the microchannels being arranged generally radially. Alternatively the rotatable support member may serve as a support for one or more devices having parallel microchannels.

15   The advantage of a common source of fluid entry force for all of the microchannels is that simultaneous filling may be ensured, the fluid samples being prevented from entering the microchannels by the hydrophobic gate means until the fluid entry force is applied. Furthermore, the
20 degree of fluid entry force may also readily be controlled, to ensure rapid filling of the microchannels, and adequate mixing. The microchannels may also be emptied in an efficient and rapid manner, by application of an increased force to the fluid in the channels, for example by
25 increasing the degree of aspiration, or by increasing the rate of rotation of the rotation support member. An exact end point of an assay may thereby be achieved. In many instances it is advantageous for the sample to be expelled before monitoring for bound target species.

30   If desired, a liquid reagent or a washing fluid may be supplied in a sealed cavity forming a reservoir, there preferably being one such reservoir per microchannel. The reservoirs may be arranged to communicate with their respective microchannels via normally closed valves, and may
35 be caused to expel their contents through such valves when acted upon by respective pistons. Alternatively, there may

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be a single reservoir, communicating via a normally closed
valve with a common conduit feeding all microchannles.

      Detection of target species with the microchannels may
be achieved by conventional means. For example, to permit
5 electrochemical   detection,   preferred   embodiments   of
apparatus are constructed so that at least a portion of the
surface of the microchannel is formed of an electrically
conductive material. This may for example be a conductive
polymer material or an electrode. In some embodiments, at
10 least a portion of the microchannel walls may be formed of
a semi-conductor material such as indium oxide. Preferably,
the semi-conductor material is transparent. Alternatively,
detection may be achieved by luminesce or fluorescence
means, in which case an electromagnetic radiation detector,
15 such as a photodiode or a photomultiplier, is provided.

      One particular advantage of the invention is that
chemical reagents may be immobilised on the inner surfaces
of the microchannels, thus providing the possibility of
ELISA - type assays in a μ-TAS-type system. A number of
20 different types of reagent may be attached to the
microchannel walls, for example oligonucleotides,
polypeptides, proteins (such as enzymes), or other natural
or synthetic molecules. Conveniently, these may be adsorbed
onto the surface of the microchannel walls, or covalently
25 linked thereto, (for example by means of amide bond
formation with succinimide), or electrostatically linked
thereto (for example by means of a crosslinker such as
polylysine). The inner surface of the microchannel and/or
of the fluid inflow channel may also be provided with
30 chemically functional groups formed by chemical or physical
treatment.

      The invention also extends to a method of manufacturing
an apparatus as defined above, comprising the following
steps which may be performed in either order or
35 simultaneously: forming at least one reaction chamber; and
forming at least one fluid inflow channel communicating with

the reaction chamber(s), at least a portion of the or each fluid inflow channel having a hydrophobic inner surface adapted to act as gate means to prevent passage of fluid through the fluid inflow channel into the reaction

5 chamber(s) until such fluid is acted upon by a fluid entry force.

For ease of fabrication, the apparatus is preferably formed in two main parts: a substrate in which the microchannels (and possibly also the inflow channels) are

10 formed as depressions (for example by injection moulding, hot embossing, photoablation, casting or polymerisation on a mould); and an overlying layer applied over the substrate and over the depressions, to form the microchannels (and optionally also the inflow channels). In embodiments in

15 which the inflow channels are not produced in the substrate they may, for example, be produced by drilling through a laminated overlying layer using a laser, or by depositing above the inlet of the reaction chamber a joint made of a hydrophobic material such as polydimethylsiloxane (PDMS).

20 The apparatus may be formed from any suitable material, for example, ceramics, glass, semiconductors, polymers, or combinations thereof. In a particularly preferred embodiment, both the substrate and lamination layer are formed of polymer material, which not only permits ready

25 formation of the microchannels (for example by photoablation), but also allows the two components to be fused together by a thermal lamination technique. For this purpose, it is preferred that at least one of the polymers is of a material which has a relatively low melting point,

30 for example polyethylene with a melting point of under 200°C. The lamination layer may with advantage be of an elastomeric material, such as polydimethyl siloxane (PDMS). In apparatus for use in conjunction with optical detection means, it is preferred that the lamination layer be formed

35 of a substantially transparent material, and the substrate of a substantially opaque material (such as a ceramics material or a carbon-filled polymer).

In another aspect, the invention extends to a method of operating an apparatus as defined, comprising the steps of: placing at least one sample of an aqueous solution under test at the end of at least one fluid inflow channel distal

5 at least one reaction chamber; causing the sample to enter the reaction chamber(s) via the fluid inflow channel(s) by applying a fluid entry force; and monitoring the sample in the reaction chamber(s) for the presence or concentration of a target substance.

10   In embodiments of apparatus having aspiration means, the fluid entry force is preferably applied by activating the aspiration means to apply reduced pressure to the microchannels for a period of time in the range 0.1 to 100s. In order to evacuate the microchannels, the aspiration means

15 may then be activated to provide an even lower pressure to the microchannels, optionally in conjunction with the supply of washing fluid from a reservoir.

In embodiments of apparatus constructed using rotatable substrates or supports, the fluid entry force is preferably

20 applied by rotating the substrate or support at an angular velocity in the range 1 to 1,000 revolutions per minute for a period of time in the range 1 to 100s. The microchannels may then be evacuated by rotating the substrate or support at an increased angular velocity, in the range 10 to 100,000

25 revolutions per minute, for a period of time in the range 1 to 100s.

The invention is hereinafter described in more detail by way of example only, with reference to the accompanying drawings, in which:

30   Fig 1 is a schematic cross section of an embodiment of apparatus according to the invention illustrated a) after deposition of an aqueous sample drop on the hydrophobic gate, and b) after sample loading;

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Fig 2 is a schematic plan view of an embodiment of apparatus according to the invention, illustrating the steps of parallel sampling, loading and washing, achieved by aspiration;

5   Fig 3 is a schematic plan view of an alternative embodiment of apparatus according to the invention, in which parallel filling and washing step are achieved by centrifugal force;

Fig 4 is a schematic plan view of a further embodiment
10 of apparatus according to the invention, in which the solution is loaded by slight aspiration and washed by strong aspiration;

Fig 5a is a partial plan view of an embodiment of apparatus according to the invention, incorporating a fluid
15 reservoir adjacent the fluid inflow channel;

Fig 5b consists of two vertical cross sections of the Fig. 5a apparatus together with an associated piston, illustrating the action of the piston in penetrating the sealed reservoir and expelling its contents through a valve
20 into the reaction chamber;

Fig 6 is a top plan representation of an embodiment of apparatus according to the invention manufactured by UV-Laser photoablation of a polycarbonate compact disk, the embodiment being constructed substantially as the apparatus
25 of Fig 3;

Fig 7 is a calibration curve obtained from a fluorescence imager using ALP-DDi solution in the microchannel of an embodiment of apparatus according to the invention;

30   Fig 8 is a graph illustrating fluorescence results indicating the level of binding between DDi-ALP in a test involving use of two microchannels of apparatus according to

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the invention, one incubated solely with BSA, and the other with BSA and anti-DDi (noted as BSA + Ab in the figure);

Fig 9 is a fluorescence image obtained using apparatus of the type illustrated in Fig 6, in which different microchannels were incubated with different concentrations of DDi-ALP;

Fig 10 is a graph illustrating the intensity of fluorescence obtained from different concentrations of ALP-DDi in an apparatus of the type illustrated in Fig 6, having antibody-coated microchannels;

Fig 11 is a graph illustrating variation of fluorescence intensity with time from an incubation of ALP-DDi in an embodiment of apparatus according to the invention, having at least one antibody coated microchannel; and

Fig 12 is a graph illustrating fluorescence intensity (indicating bound ALP-DDi) in a competitive immunoassay with D-Dimer utilising an embodiment of apparatus according to the invention, having at least one antibody-coated microchannel.

The microchannel devices of Figs 1 to 6 are produced by UV-Laser photoablation of commercially available polymers such as PET or polycarbonate. The photoablation procedure is performed in known fashion, for example as described previously by the present applicants[44]. Briefly, a polymer sheet is rinsed with distilled water and ethanol and then mounted on an X, Y machining stage (Microcontrol, France). UV-Laser pulses (193 nm) (Lambda Physik LPX 205 i, Germany) are then fired at the polymer substrate target through a photomask and a 10:1 lense with a frequency of 50 Hz at 200 mJ/pulse, corresponding to a fluence per pulse of 1 J/cm^2 on the surface. During the photoablation process, the polymer substrate is moved horizontally with a X, Y stepping motor (Microcontrol, France) at a speed of 0.2 mm/s resulting in

linear channels 22 mm long. The microchannels are typically between 1 and 1,000µm in width, and in this example are approximately 100µm wide. The depth of the channels was fixed at 40 µm, by controlling the number of laser pulses
5   used (each pulse photoablates approximately 150 nm). The channels are then sealed by thermal lamination of a layer of polyethylene over the base polymer sheet, the channels then exhibiting a trapezoidal shape in which three walls are composed of the substrate polymer (PET or Polycarbonate) and
10 the top is composed of the lamination (Polyethylene). Fluid inflow channels (or "gates") (1) are opened either by firing enough laser pulses or are mechanically drilled through the hydrophobic lamination layer. The gates, which may have a diameter between 10µm and 10mm, have hydrophobic inner
15 surfaces due to the nature of the polymer, and therefore inhibit passage of aqueous fluids.

    The precise arrangement of microchannels is not crucial to the operation of the invention, though two general geometries have been developed and tested by the applicants
20 and proved to be of benefit. In the first of these, a plurality of microchannels are arranged parallel to each other, conveniently on a generally rectangular substrate. The inflow channel "gates" of the various microchannels are aligned with each other, to permit rapid and efficient
25 loading with test solutions from a linear multiple pipette device (see Fig 2). In the second configuration, the microchannels are arranged radially on a generally circular substrate, either with the inflow channel gates towards the centre of the circle and the opposite (outflow) ends of the
30 microchannels towards the circumference (Figs 3 and 6), or vice versa (Fig 4).

    A number of different means may be employed to provide the fluid entry force, of which the preferred means are aspiration and centrifugal force. In the apparatus of Fig
35 2, a common conduit (3) is supplied at the outflow ends of the microchannels (2), to which a reduced pressure is applied during operation of the device, to draw fluid into

the microchannels through the fluid inflow gates. As
illustrated in the last representation in Fig 2, the
aspiration means may also be utilised to supply a stronger
aspirating force in order to expel the microchannel contents
5 to a drain, optionally in conjunction with the supply of a
washing fluid. The apparatus illustrated in Fig 4 operates
in a similar fashion, with aspiration being applied to the
common outflow drain (6). In the apparatus illustrated in
Figs 3 and 6, fluid is compelled to pass through the fluid
10 inflow gates and into the microchannels by spinning the
substrate to produce centrifugal force. In the illustrated
arrangement, each microchannel has its own drain (7).

    Typically, in an aspiration driven device (as in Figs
2 and 4) a 2 μl sample is placed with a pipette on each gate
15 (1). The solution is then loaded into the microchannel by
a brief aspiration from the common conduit (3;6). This
technique ensures homogeneity of the solution over the whole
microchannel. After incubation, the microchannel is
aspirated and rinsed three times with 2 μl. It is worth
20 noting that the washing solution volume is much larger than
that of the microchannel (about 100 nl) thus ensuring
efficient washing. Using devices driven by centrifugal
pressure, the filling and washing procedures may be achieved
by placing 2 μl of solution over each gate (1). Slow
25 rotation results in loading of the sample into the
microchannel(s), and faster rotation is subsequently used to
expel the sample from the microchannel(s).

    Fig 5 illustrates an optional modification of apparatus
according to the invention, in which each microchannel has
30 an associated fluid reservoir (10) formed by a sealed cavity
situated adjacent the fluid inflow gate (1). The reservoir
communicates with microchannels (2) by means of a normally
closed valve (12), which comprises valve member (13) which
may be deformed under pressure into depression (14).
35 Reservoir (10) is capped by seal (15), which may be broken
by downward pressure applied by piston (11), which is
profiled to be a close fit within reservoir (10). Downward

movement of piston (11) within reservoir (10) increases the
fluid pressure within the reservoir, thus opening valve (12)
and allowing fluid from the reservoir to enter the
microchannel.   Depending upon the requirements of any
5 particular assay, the reservoir may either be filled with a
reagent or with wash fluid.

By way of example, various tests were carried out to
establish the utility of apparatus according to the
invention in performing an immunoassay for D-Dimer. D-Dimer
10 is used as a diagnostic indicator in thromboembolic events:
deep vein thrombosis and pulmonary embolism can be diagnosed
by monitoring D-Dimer concentration in blood. In the past,
the most reliable assay of D-Dimer have been performed by
ELISA techniques, for example the "Asserachrom D-Di" of
15 Diagnostica Stago. However, standard ELISA techniques are
not suited for emergency situations, and alternative
membrane-based techniques have been developed which use
colour based detection systems [48]. However, these suffer from
the disadvantage that the detection mechanism is too
20 subjective.

In the present tests, the detection of the enzyme was
effected by a chemifluorescent substrate solution (VCR,
Amersham).   This system is based on the fluorescent
detection of the AttoPhos substrate hydrolysed by ALP. The
25 microchannels were then exposed to a Fluorescence Imager
screen (MP840, Molecular Dynamics) and every channel was
read for 1 minute. The image was then quantified using
Image Quant software (Molecular Dynamics). The calibration
of the enzyme in the microchannel was achieved by mixing the
30 substrate solution with different concentrations of enzyme
and incubating for 5 minutes. The microchannels were then
filled with the mixtures and analysed with the fluorescence
imager. In the actual tests, the enzyme was immobilised on
the surface of the microchannels, and the VCR solution was
35 added to the channels with fluorescence being measured 5
minutes later.

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Immobilisation of the proteins was achieved by physisorption for 1 hour at room temperature. The mouse IgG antibody (Serbio, France) was immobilised by placing either 10 or 100 µg/ml in the microchannel, followed by incubation
5 for 1 hour in a wet chamber. The surface was then washed with PBS and 20 % Tween (Tween/Water :0.2 ml/L, Fischer Germany), and blocked for 1 hour with a solution of 50 µg/ml of heat shocked BSA (Sigma, USA) in the washing buffer solution. After another washing step, the channels were
10 individually filled with the antigen solution. After five minutes (except for the kinetic experiment, where other periods are specified below), the microchannels were rinsed and a solution of 10 µg/ml of alkaline phosphatase labelled antigen (ALP-DDi) was introduced and rinsed again after five
15 minutes.

Fluorescence dependence on enzyme concentration after 5 minutes of incubation is presented graphically in Figure 7. The detection limit is reached in the range of 1 ng.ml^{-1}. The non-linear detection range is due to the fact that the
20 product of the hydrolysis is not highly soluble and may precipitate on the surface at higher concentrations. Nevertheless, this system can be used for quantifying the enzyme concentration in the microchannel.

In order to study the activity of the adsorbed
25 antibodies in the microchannel, two different incubation procedures were undertaken. Firstly, a few channels were incubated only with BSA. Secondly, some further channels were incubated with Ab and then with BSA. Every channel was then filled with the DDi-ALP, incubated for 1 hour and
30 washed by aspiration following the procedure described above. The fluorescence intensity of each channel was then measured and the results are presented in Figure 8. The channels incubated only with BSA exhibited a low fluorescence which is not significantly different from that
35 of the polymer substrate itself. In contrast the channels incubated with the antibodies were much more fluorescent, thereby demonstrating that DDi-ALP was adsorbed on the

antibodies. This experiment shows that some of the adsorbed
antibodies are still active on the surface and that BSA is
an effective blocker against the non-specific adsorption of
the DDi-ALP complex.

5   Figure 9 shows the fluorescence of the substrate in the
channels after adsorption of different concentrations of
ALP-DDi on the 10 $\mu g.ml^{-1}$ adsorbed antibodies. The
fluorescence intensity of the microchannel lines clearly
shows the gradient of concentration in the different
10 microchannels. The relative intensity of every microchannel
is shown graphically in Figure 10. Saturation of channels is
reached at about 30 $\mu g/ml$.

     Figure 11 shows the fluorescence intensity of
microchannels that have been incubated for different periods
15 of time. For short incubation times (<5 min), the intensity
grows linearly, showing that the antigens are very quickly
captured by the antibodies. It is thought that all the
antigens have still not reached the surface by diffusion.
This first slope approximately follows the diffusion of the
20 molecules to the walls. The molecules then react rapidly and
the reaction becomes quasi diffusion-controlled. After 5
minutes of incubation, the reaction is controlled by slower
kinetics driven by two different phenomena. Firstly, large
molecules diffuse much more slowly and therefore reach the
25 surface after a long time. In this case, the molecules can
be partially degraded fibrin products, of which the
molecular weight can be larger that 1000 kD. Secondly, there
is a tendency for non-specific adsorption; such reactions
are much slower than immunological recognition and are
30 driven by electrostatic or hydrophobic interactions that
require reorganisation at the molecular level. This type of
non specific adsorption may therefore be excluded by short
incubation times.

     Figure 12 shows the fluorescence dependence of the D-
35 Dimer concentration after a competitive immunoassay. In the
low concentration range, most of the immobilised antibody

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sites are not occupied by the DDi, allowing the DDi-ALP to be present in a large amount and therefore to hydrolyse more fluorescent substrates. At concentrations higher than 1000 ng.ml^{-1}, D-Dimer molecules are present on most of the
5 antibody sites and therefore only a few sites are available for DDi-ALP. The central part of the concentration range (100-1000 ng.ml^{-1}) shows the strong concentration dependence of the system, the two orders of magnitude detection range being in the range of interest for diagnostics applications
10 [49].

These experiments demonstrate the feasibility of ELISA techniques in microchannels. Benefitting from fast equilibration times and rapid filling and rinsing procedures, the time taken to complete an assay (including
15 calibration) may be reduced to less than 10 minutes, compared to a typical time of 3 hours for an ELISA in a microtiter plate. Hundreds of microchannels may be provided on a substrate, if desired, and the ability to ensure simultaneous filling provides the possibility of highly
20 efficient parallel assays.